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Binding Interactions between Two Ligands and a Monomeric Protein

Study on Indole, Protons and Chymotrypsin

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The binding of indole and protons to chymotrypsin has been studied by polarimetry and potentiometry.

δ-Chymotrypsin is able to assume two discrete conformations E and E′. The conversion of E′ to E can result from the binding of a proton to the α-amino group of the N-terminal isoleucine number 16, from the binding of a molecule of indole to the active site of the enzyme, or from the binding of both ligands. These two conformations are in equilibrium; the equilibrium is completely shifted by the binding of these ligands to their specific site. In the absence of indole the E conformation is maintained by the NH_3^+–COO^- interaction between isoleucine 16 and aspartic acid 194. In the absence of the proton on this isoleucine residue the E conformation is maintained by indole. The protonation of the isoleucine 16 residue decreases the binding constant of indole and the presence of indole decreases the binding constant of the proton. The pK of isoleucine 16 is shifted from less than eight in the E′ state to 10·5 in the E state and the conversion of E′ to E upon binding indole is accompanied by a proton uptake. These results are correlated with the crystallographic data available for chymotrypsin and with the similar transition observed at acidic pH.

1. Introduction

Binding interactions between substrate molecules or between substrate and effectors have been extensively studied since the discovery of allosteric enzymes. In all cases, whether the interaction is homotropic or heterotropic, the effect is expected to be related to a conformational transition of the protein; it implies that in the case of heterotropic interactions, the effector, a compound of specific structure, binds to a specific site of complementary geometry on the enzyme surface. However, no limitation in size is considered by any of the models (Monod, Wyman & Changeux, 1965; Koshland, Nemethy & Filmer, 1966), and an heterotropic effector can be as small as a proton. This is found in the Bohr effect (Bohr, Hasselbalch & Krogh, 1904; Antonini, 1965). Furthermore, as noted in both theories, heterotropic effects can, in principle, be observed for monomeric proteins as well as oligomeric proteins; thus, a Bohr effect is visible in lamprey haemoglobin (Antonini, Wyman, Bellelli & Rumen, 1964).

Therefore one can ask the question whether pH-dependence of enzyme activity cannot be considered in some instances as both a simple and extreme case of heterotropic allosteric interaction. The rather universal control of enzyme activity by pH can indeed be independent of any catalytic step *per se*; in such a case it reflects a more

or less reversible structural change of the protein. This dependence would be an example of binding interaction providing that:

(1) binding of the proton and of the substrate mutually affect each other through a conformational change of the protein;

(2) it is due to the fixation of one (or very few) proton(s) on a well-defined locus of the enzyme polypeptide chain.

Chymotrypsin affords a good model for studying such a phenomenon. Its catalytic activity is known to decrease above pH 8 for those substrates that have an apparent acylation rate limiting step (Bender, Clement, Kezdy & d'A. Heck, 1964) and it has been shown that the actual controlling factor is substrate binding (Himoe, Parks & Hess, 1967). As there is no relationship between substrate (e.g. *N*-acetyl tryptophan amide) ionization and pH, in the range pH 8 to 11, any change in substrate binding has to be related to the enzyme. Protonation of the N-terminal isoleucine residue number 16 of the polypeptide chain B of the enzyme is indeed known specifically to control the structure of the protein (Oppenheimer, Labouesse & Hess, 1966; Ghélis, Labouesse & Labouesse, 1967; Matthews, Sigler, Henderson & Blow, 1967; Karibian, Laurent, Labouesse & Labouesse, 1968). Therefore one may expect some relationship between these different phenomena.

The fact that covalent binding of a pseudo-substrate produces a modification of the protein flexibility had already been suggested by the observation that diisopropyl-chymotrypsin undergoes no change in optical rotation between pH 8 and 10 whereas the free enzyme does (Parker & Lumry, 1963; Havsteen & Hess, 1963). However, acylation of the enzyme-active serine residue by diisopropylfluorophosphate cannot be considered as a binding step controlled by the law of mass action since it is irreversible. Therefore the question is raised whether reversible binding of a non-covalent specific inhibitor would be also pH and conformation dependent, i.e. whether there is a relationship between binding of one molecule of inhibitor and of one proton to the enzyme.

This work presents data showing the relationship between binding of a non-polar specific inhibitor, indole, binding of one proton and changes in the protein conformation, with acetylated-δ-chymotrypsin.

2. Materials and Methods

Chymotrypsinogen, which had been crystallized 3 times was obtained from Worthington Biochemical Corp. Acetylated-δ-chymotrypsin was prepared as described by Oppenheimer *et al.* (1966). Protein concentration was determined by measuring the absorbance at 280 nm using a molar extinction coefficient of $E_{280} = 4{\cdot}7 \times 10^4$ for the protein in which the exposed tyrosines are acetylated, and $E_{280} = 5 \times 10^4$ for the protein in which the exposed tyrosines are unacetylated (Oppenheimer *et al.*, 1966). Enzyme activity was checked in a pH-stat according to Schwert & Takenaka (1955) using *N*-acetyl-L-tyrosine ethyl ester as substrate. The indole binding constant was measured by inhibition of *N*-acetyl-L-tyrosine ethyl ester hydrolysis at 15°C and pH 7·8.

Optical rotation measurements were carried out in a Sofica Spectropol spectropolarimeter, using a 1 dm thermostatically controlled cell. Before the measurements were made, the enzyme solutions were centrifuged for 1 hr at 30,000 *g*. The enzyme was allowed to equilibrate in a thermostatically controlled bath, then indole and buffer were added and the optical rotation recorded. The final concentrations in the cell were: enzyme, 1·0 to 1·5 mg/ml.; indole, 0 to 9 mM; acetonitrile, 1%. Phosphate, borate and carbonate buffers, 0·02 M, were used according to pH. Temperature was 15°C and ionic strength adjusted to $\mu = 0{\cdot}15$ with KCl. For each measurement, the optical rotation was extrapolated to zero time, because of the deacetylation of tyrosine (Karibian *et al.*, 1968). The chosen wave-

length was 320 nm as this gave the most accurate results but it was checked that the same phenomena occur in the optical rotatory dispersion spectrum, from 310 to 500 nm. Optical rotation was measured with an accuracy of $\pm$ 0·002°.

Potentiometric titrations and proton absorption measurements were performed with an Eil Vibron pH meter. Unbuffered enzyme solutions were obtained by exhaustive dialysis against 0·15 M-KCl prepared with boiled water and protected against carbon dioxide absorption. A 10-ml. enzyme sample containing 15 to 20 mg Ac-δ-CT†, was titrated in a jacketed cell, under nitrogen. Titrations were performed with 0·02 M-HCl and KOH, reversibility being checked after each run. Because of tyrosine deacetylation, titrations had to be stopped around pH 10. For proton absorption experiments, the pH of the enzyme sample was adjusted to the final value, then 0·5 ml. of concentrated solution of indole in 50% acetonitrile was added, so that the final concentration of acetonitrile was always 2·5%. The quantity of 0·02 M-HCl required to bring the pH back to its initial value was measured with an Agla-microsyringe. In the titration experiments, acetylated chymotrypsinogen in the presence of indole and acetonitrile was used as a blank. It was checked that acetonitrile alone did not induce any significant proton uptake with Ac-δ-CT, and then Ac-δ-CT with acetonitrile alone was used as a blank in proton absorption measurements.

All the data were obtained with Ac-δ-CT. As previously discussed (Himoe *et al.*, 1967; Karibian *et al.*, 1968) they are considered to be relevant to δ-chymotrypsin.

3. Results

(a) *Conformational change associated with binding of protons and indole*

It has been previously shown (Oppenheimer *et al.*, 1966; Karibian *et al.*, 1968) that the optical rotation of chymotrypsin is pH-dependent in the range of pH 7·5 to 10·5, and that the equilibrium between the two protein conformations is displaced by the ionization of a single group, the α-amino group of the N-terminal isoleucine residue of the B chain (no. 16) (Ghélis *et al.*, 1967). As it was pointed out by Tanford (1961), in the case of a conformational change linked to the ionization of one group, the pH dependence curve of the observed structural parameter is identical to the titration curve of this group. This pH-dependent transition corresponds to the apparent equilibrium:

$$\text{(I)} \qquad EH \overset{K_a}{\rightleftharpoons} E' + H$$

where E is the conformation prevailing at neutral pH and E′ that at alkaline pH. If $|\alpha|$ is the specific rotation observed at neutral pH and $|\alpha| + \Delta_m$ that observed at alkaline pH, then at each pH value, the observed specific rotation Δ_0 will be given by:

$$\Delta_0 = \Delta_m \frac{K_a}{H + K_a}. \qquad (1)$$

From Figure 1, it can be found that the value of K_a is $(1 \pm 0{\cdot}1) \times 10^{-9}$ M.

Figure 2 shows that a similar behaviour occurs for Ac-δ-CT in the presence of the competitive inhibitor indole. However the sigmoidal curves of the specific rotation of the protein, $|\alpha|$, *versus* pH are shifted towards higher pH values and the upper plateaux are lowered, the extent of both these effects increases with indole concentration.

At neutral pH, indole binding does not seem to have any measurable effect on the enzyme conformation (see Fig. 2), so that the binding equilibrium is:

$$\text{(II)} \qquad EH + I \overset{K_i}{\rightleftharpoons} EIH$$

† Abbreviation used: Ac-δ-CT: acetylated-δ-chymotrypsin, a derivative in which all ε-amino groups of lysines, the α-amino group of the N-terminal half cystine and the phenolic groups of the two titratable tyrosines are acetylated.

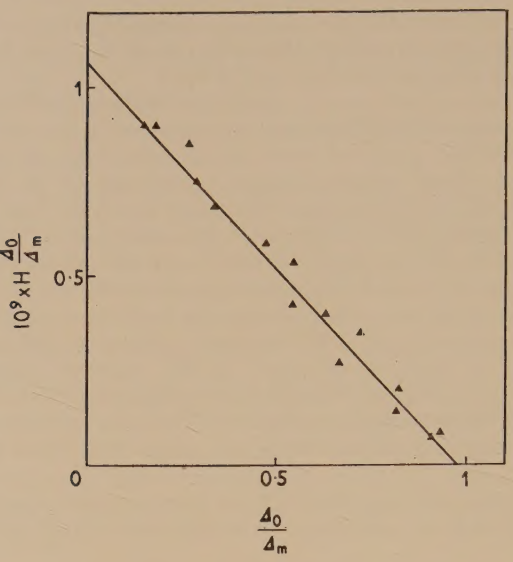


FIG. 1. The pH-dependence of specific optical rotation change, Δ_o, of Ac-δ-CT in the absence of indole; Δ_m is the maximum rotation change. Conditions: enzyme 1 to 1·5 mg/ml., buffer 2×10^{-2} M, ionic strength $\mu = 0{\cdot}15$, temperature 15°C, wavelength 320 nm.

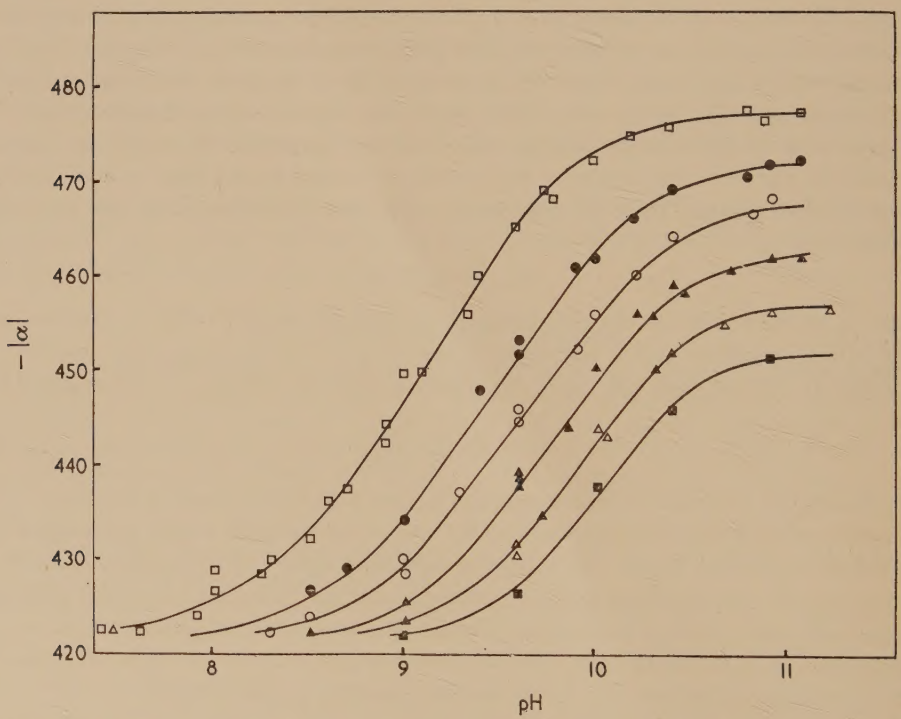


FIG. 2. The pH-dependence of the specific optical rotation $|\alpha|$, at 320 nm of Ac-δ-CT obtained for different indole concentrations: (□)$I = 0$; (●)$I = 10^{-3}$ M; (○)$I = 2 \times 10^{-3}$ M; (▲)$I = 4 \times 10^{-3}$ M; (△)$I = 6 \times 10^{-3}$ M; (■)$I = 9 \times 10^{-3}$ M. Conditions: enzyme 1 to 1·5 mg/ml., buffer 2×10^{-2} M, ionic strength $\mu = 0{\cdot}15$, acetonitrile 1% (v/v), temperature 15°C. The curve corresponding to $I = 0$ was used in Fig. 1.

where the enzyme conformation, as seen from optical rotation, is the same in the two species. The value of K_i has been determined by inhibition of *N*-acetyl-tyrosine-ethyl-ester hydrolysis at pH 7·8 and 15°C. The value found for K_i is 5×10^{-4} M. This figure is in agreement with values found by Huang & Niemann (1953) and Yapel, Han, Lumry, Rosenberg & Schiao (1966), in slightly different conditions for α-chymotrypsin.

Above pH 8, indole binding seems to drive the enzyme conformation from the E′ form towards the E form (without taking into account any effect on proton binding). If at each pH, this polarimetric effect of indole binding is due to a saturation phenomenon that leads to a decrease in the concentration of the E′ form, as seen in Figure 2, then the semi-logarithmic plot of $|\alpha|$ *versus* pI ($= -\log I$) is expected to be sigmoidal for each pH value. Figure 3 shows a plot of $|\alpha|$ *versus* pI. Unfortunately the maximum

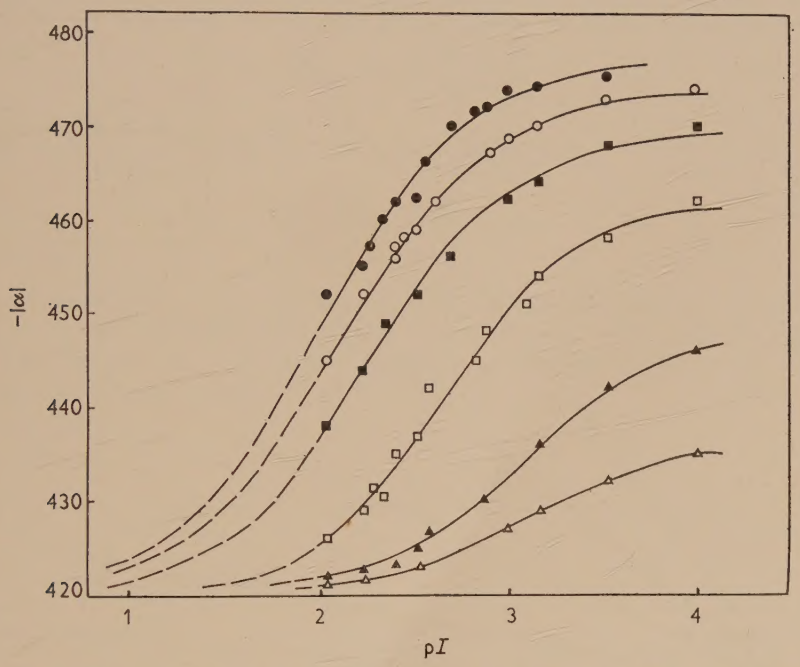


FIG. 3. The dependence of the specific optical rotation | α | at 320 nm of Ac-δ-CT in the presence of indole *versus* pI = − log I for different pH values:

(●) pH 10·9; (○) pH 10·4;
(■) pH 10; (□) pH 9·6;
(▲) pH 9; (△) pH 8·5.

Conditions as described in Fig. 2.

indole solubility (10^{-2} M at 15°C) is not high enough to saturate the enzyme. The lack of experimental results above an indole concentration of 10^{-2} M does not give direct evidence of whether or not the specific rotation of the enzyme when saturated with indole is the same as that observed when saturated with protons. Figures 2 and 3 show that upon binding H^+, indole or both, the relative amount of the E′ conformation decreases.

If $|\alpha|$ is the specific rotation of Ac-δ-CT at neutral pH, $|\alpha| + \Delta_0$ is that at any pH in absence of indole and $|\alpha| + \Delta$ is that at the same pH in presence of indole, the varia-

tions of $\Delta_0 - \Delta$ with the indole concentration I will indicate the disappearance of the E′ form brought about by indole binding. Figure 4 shows a plot of $\Delta_0/(\Delta_0 - \Delta)$ *versus* $1/I$, which is analogous to a normalized Lineweaver–Burk plot. The Michaelian behaviour of the enzyme structure suggested by Figures 2 and 3 is confirmed by the straight lines obtained, and their slopes give the apparent binding constant of indole $(K_i)_{app}$ at each pH. The intercepts of these lines with the ordinate axis are close to one, and no deviation from this value can be correlated with pH as seen in Figure 4(b). This strongly suggests that, within experimental error, the specific rotation of the indole-saturated enzyme is pH independent and is identical to that of the proton-saturated enzyme (where "proton-saturated enzyme" refers specifically to the protein in which one proton is bound to the isoleucine 16 residue).

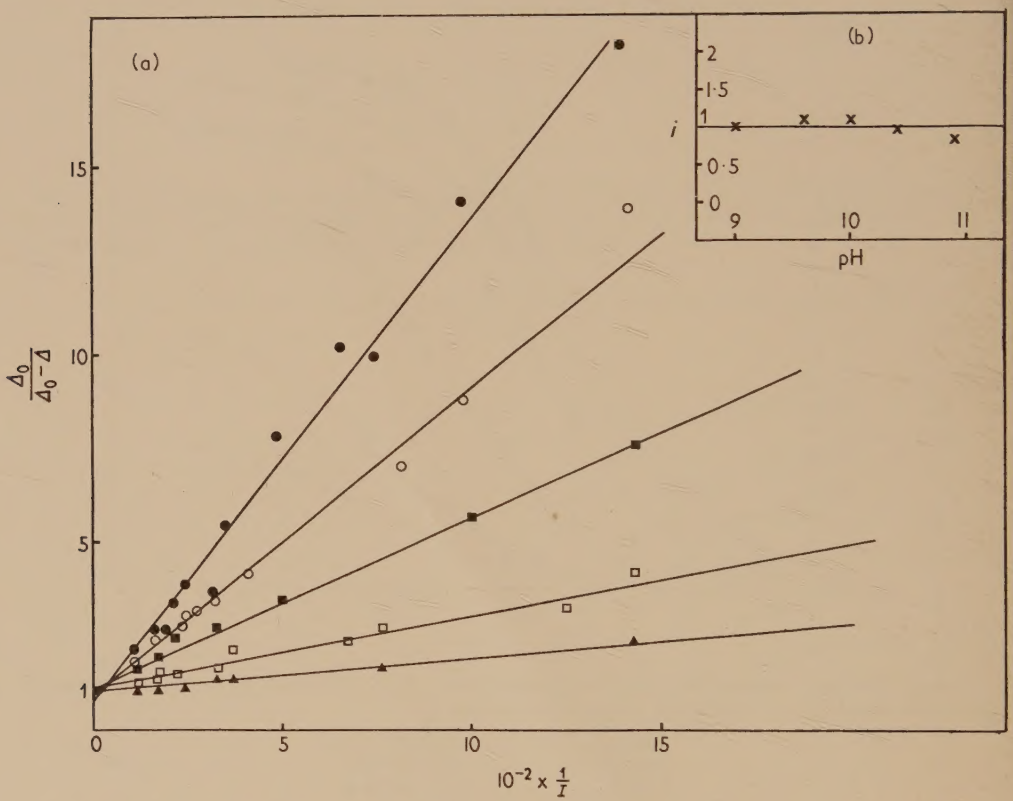


FIG. 4. (a) Double reciprocal plot of $\Delta_0/(\Delta_0 - \Delta)$ *versus* $1/I$ (see text) for several pH values. Symbols and conditions as described in Fig. 3. The solid lines were computed by the least-squares method. (b) Intercept i of the lines of Fig. 4 (a) for $1/I = 0$ as a function of pH.

If we assume the identity of the structures of the E form and of the indole–protein complex, we can investigate the ionization state of the α-amino group of isoleucine 16 in this complex. There are two possible explanations for the fact that optical rotation of the indole–protein complex seems to be pH independent:

(i) The pK of this amino group in the complex is shifted to a very alkaline value, out of the investigated pH range, as proposed by Bender & Wedler (1967).

(ii) The pK of this amino group belongs to the investigated pH range, but the ionization of this group takes place without detectable structural variations.

Both cases can be described by the following scheme:

$$\text{(III)} \qquad \begin{array}{ccc} EH + I & \overset{K_a}{\rightleftharpoons} & E' + H + I \\ {\scriptstyle K_i}\uparrow\downarrow & & \uparrow\downarrow{\scriptstyle K'_i} \\ EHI & \overset{K'_a}{\rightleftharpoons} & (EI \text{ or } E'I) + H \end{array}$$

With hypothesis (i), one cannot ascertain whether the high pH saturated enzyme has the E or the E′ conformation, since this species would be present in negligible concentration (K'_a very small). If K'_a is experimentally accessible (hypothesis (ii)), the indole saturated enzyme has the E conformation at alkaline pH.

This scheme gives a general expression for the apparent binding constant of indole:

$$(K_i)_{app} = K_i \frac{H + K_a}{H + K'_a} \tag{2}$$

thus a plot of $\dfrac{H + K_a}{(K_i)_{app}}$ *versus* H is expected to be linear:

$$\frac{H + K_a}{(K_i)_{app}} = \frac{1}{K_i}(H + K'_a). \tag{3}$$

Figure 5 shows such a plot; the slope is $1/K_i$ and the extrapolation on the abscissa axis is $-K'_a$. The value found for K'_a is 4×10^{-11} M, (p$K'_a = 10{\cdot}4$). The slope gives an equilibrium constant $K_i = 5{\cdot}5 \times 10^{-4}$ M which agrees with the kinetically obtained inhibition constant $K_i = 5 \times 10^{-4}$ M.

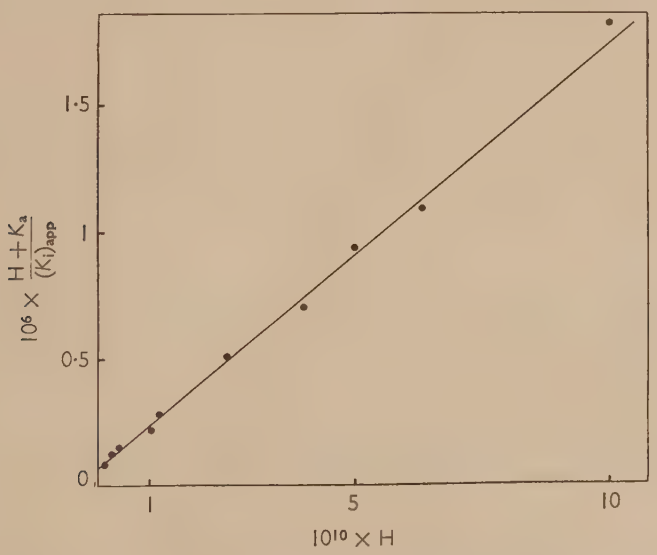


FIG. 5. Plot of $(H+K_a)/(K_i)_{app}$ *versus* H for the determination of K'_a. The values of $(K_i)_{app}$ were determined from the slopes of the straight lines of Fig. 4 and of similar data. The solid line was determined by least-squares regression.

Therefore a better scheme seems to be:

$$\text{(IV)}\qquad \begin{array}{ccc} \mathrm{EH} + \mathrm{I} & \underset{}{\overset{K_a = 10^{-9}\,\mathrm{M}}{\rightleftharpoons}} & \mathrm{E'} + \mathrm{H} + \mathrm{I} \\ {\scriptstyle K_i = 5\times10^{-4}\,\mathrm{M}}\uparrow\downarrow & & \uparrow\downarrow {\scriptstyle K'_i = 1{\cdot}5\times10^{-2}\,\mathrm{M}} \\ \mathrm{EHI} & \underset{K'_a = 4\times10^{-11}\,\mathrm{M}}{\rightleftharpoons} & \mathrm{EI} + \mathrm{H} \end{array}$$

The complex inhibition constant $K'_i = (K_a . K_i)/K'_a$ corresponds, like K_a, to both an isomerization step and a dissociation step. Its value is $K'_i \sim 1{\cdot}5 \times 10^{-2}$ M.

(b) *Proton binding displacement associated with indole binding*

If a change of pH shifts the apparent binding constant of indole, then a change of indole concentration should modify the apparent pK of the N-terminal amino group.

Figure 6 shows the potentiometric titration curves of this amino group in the free enzyme and in the presence of indole. The apparent pK of isoleucine 16 has been found

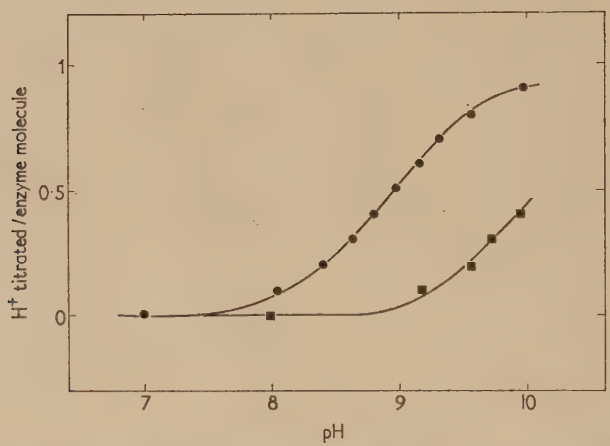


FIG. 6. Difference titration curves of Ac-δ-CT *versus* acetylated chymotrypsinogen in absence of indole (●), and in presence of 5×10^{-3} M-indole (■), giving the number of protons titrated per enzyme molecule in the pH range 8 to 10. These curves are the mean of experiments performed with different enzyme concentrations from 1·5 to 2 mg/ml.; other conditions: acetonitrile 2·5% (v/v), temperature 15°C, ionic strength $\mu = 0{\cdot}15$.

to be 8·9 ± 0·1 in good agreement with the previous polarimetric and kinetic measurements (Karibian *et al.*, 1968). In the presence of indole, the pK of this amino group seems to be shifted towards higher pH values. However these titrations could not be carried out above pH 10 because of the deacetylation of tyrosine, and thus they could not give quantitative measurements.

Considering scheme (IV), it is possible to calculate the protonated fraction f of the protein at each pH and indole concentration:

$$f = \frac{\mathrm{EH} + \mathrm{EHI}}{\mathrm{EH} + \mathrm{EHI} + \mathrm{EI} + \mathrm{E'}} = \frac{\mathrm{H}\,(\mathrm{I} + K_i)}{(\mathrm{H} + K_a)K_i + (\mathrm{H} + K'_a)\mathrm{I}} \qquad (4)$$

and the difference p in protein bound protons when increasing indole concentration from zero to I (the subscript $_0$ referring to absence of indole):

$$p = f_0 - f = \frac{H}{H + K_a} - \frac{H(I + K_i)}{(H + K_a)K_i + (H + K'_a)I}; \quad (5)$$

at indole saturation:

$$p_{max} = \frac{H}{H + K_a} - \frac{H}{H + K'_a}. \quad (6)$$

Equation (5) shows that adding indole results in a proton uptake in the pH range $K_a > H > K'_a$; this proton uptake is zero for $H \ll K'_a$ or $H \gg K_a$. Therefore a maximum proton uptake has to be observed at some intermediate pH. Differentiating equation (5) gives this pH:

$$pH_{max} = pK_a + \frac{1}{2}\log\frac{K_i + I}{K'_i + I} + \frac{1}{2}\log\frac{K'_i}{K_i} \quad (7)$$

which shows that at saturation in indole ($I = \infty$) this pH is given by:

$$pH_{max} = \frac{1}{2}(pK_a + pK'_a) \quad (8)$$

as already proposed by Glick (1968).

With the values found for $K_a = 10^{-9}$ M and $K'_a = 4 \times 10^{-11}$ M, the maximum proton uptake is expected to be 0·7 proton per enzyme molecule at pH 9·7.

This proton uptake upon addition of indole was measured at different pH and indole concentrations, then extrapolated for each pH value to saturation in indole by use of a double reciprocal plot of $1/p$ *versus* $1/I$. This plot was found to be linear as expected from equation (5), as shown in Figure 7. This plot gives the proton uptake at indole

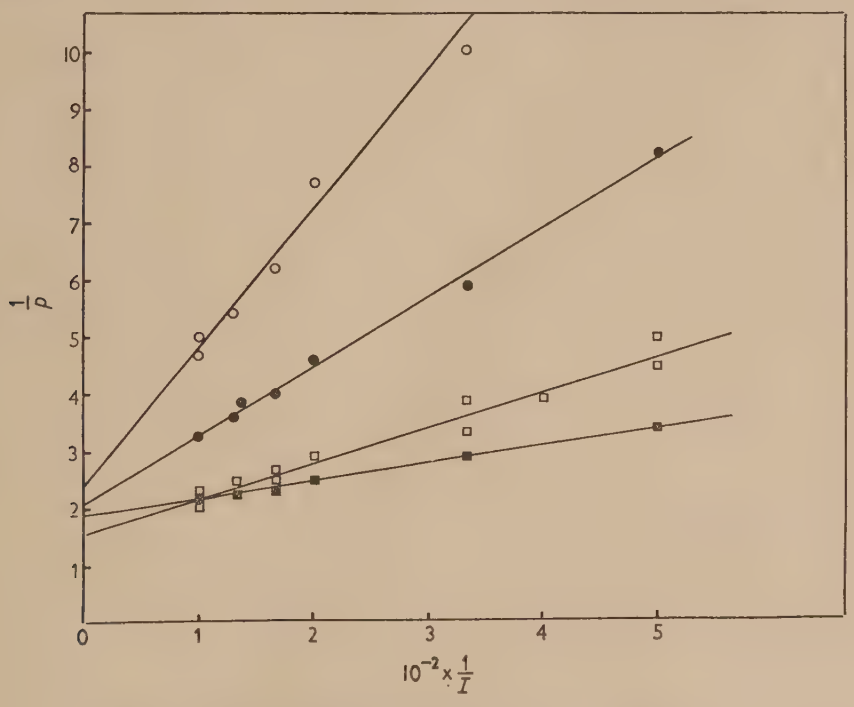


FIG. 7. Double reciprocal plot of $1/p$ *versus* $1/I$ at different pH:
(○) pH 10·6; (□) pH 9·7;
(●) pH 10·2; (■) pH 9·4.
Conditions as described in text.

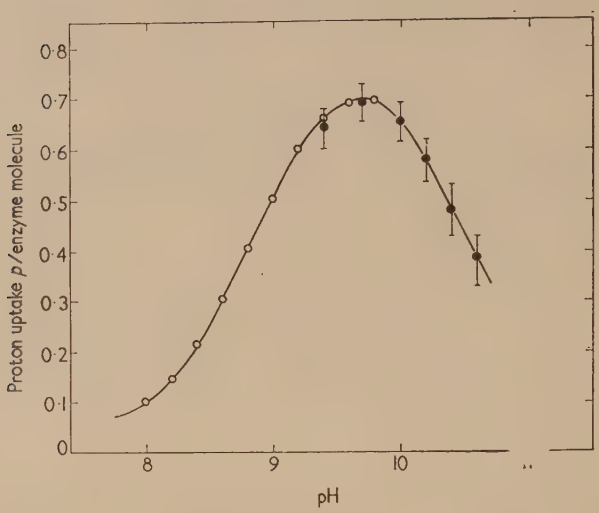


FIG. 8. The pH dependence of the proton uptake, p, by Ac-δ-CT upon increasing indole concentration from zero to saturation, as determined by direct titration (○), and by proton absorption measurements (●). The solid line is the theoretical curve corresponding to $pK_a=9$ and $pK'_a=10{\cdot}5$ as given in equation (6). Conditions as described in Fig. 6. The values of p at indole saturation were taken from the data used in Fig. 7.

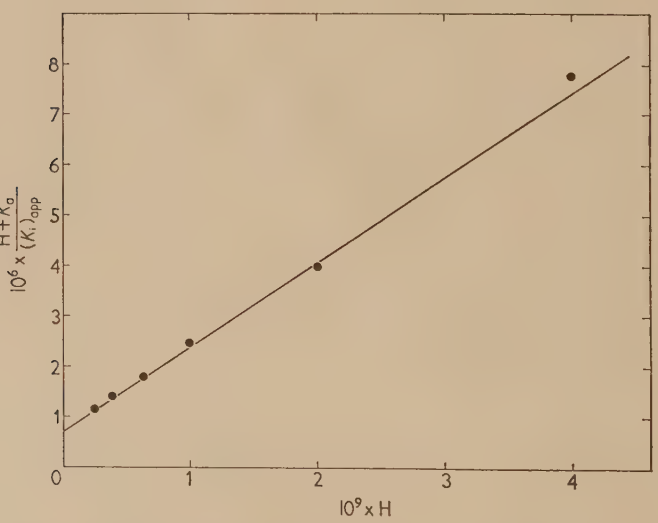


FIG. 9. Plot of $(H + K_a)/(K_i)_{app}$ for values of $(K_i)_{app}$ obtained from proton absorption measurements. $(K_i)_{app}$ was taken from the slopes of the lines in Fig. 7, and similar data.

saturation ($1/I = 0$) and the apparent indole binding constant $(K_I)_{app}$. Figure 8 shows the pH dependence of the proton uptake obtained for indole saturation. The bell-shaped curve obtained fits with equations (6) and (8), giving a dissociation constant $K'_a = (3 \pm 1) \times 10^{-11}$ M, close to that determined by optical rotation (4×10^{-11} M).

Using equations (5) and (6) we obtain:

$$\frac{p_{max}}{p} = 1 + \frac{H + K_a}{H + K'_a} \times \frac{K_I}{I} = 1 + \frac{(K_I)_{app}}{I}$$

where $(K_I)_{app}$ is given by the same expression as found from optical rotation measurements. Thus a plot of $H + K_a/(K_I)_{app}$ *versus* H should give a second independent determination of K'_a. This is shown on Figure 9; the value obtained is $K'_a = 3 \times 10^{-11}$ M ($pK'_a \simeq 10{\cdot}5$), and is in agreement with the value obtained by optical rotation determinations.

4. Discussion

From the data presented in this study it appears that binding interactions occur between indole, H^+ ions, and chymotrypsin, and that this interaction occurs through a conformational change of the enzyme. Figures 1 and 3 show, respectively, that one proton and one molecule of indole are involved in this interaction. It has been previously demonstrated (Karibian *et al.*, 1968. and references therein) that the critical residue on which this proton binds is the N-terminal isoleucine residue 16 of the B chain of the enzyme. This residue interacts *via* electrostatic forces, in a hydrophobic environment, with the aspartic 194 residue next to the active serine residue (Matthews *et al.*, 1967). Indole binds specifically to the substrate binding site of the enzyme (Yapel *et al.*, 1966) and the polarimetrically observed conformational change probably involves a limited part of the protein molecule, close to the active site (Sigler, Blow, Matthews & Henderson, 1968; McConn, Fasman & Hess, 1969). Therefore conditions (1) and (2) defined in the introduction to this work seem to be fulfilled.

(a) *Equilibrium between two conformations of the enzyme*

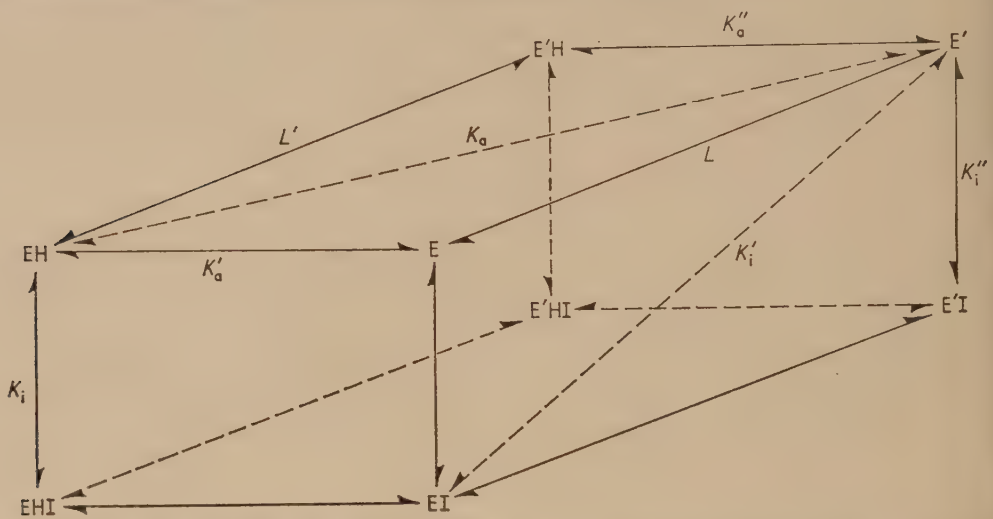
At neutral pH, the fact that no significant change in optical rotation could be observed upon addition of indole, seems to indicate that the protonated form of the enzyme does not undergo part of the change in conformation that is observed at high pH when binding this ligand; if no further conformational transition occurs, one can conclude that there is a negligible amount of the E'H or E' species at neutral pH, and that any secondary structural effect of indole binding on the EH species is not detected by polarimetric measurement. At alkaline pH two types of protein molecules can be considered: those that have not bound indole; those that have bound an indole molecule. In the absence of indole the polarimetric and the potentiometric titration curves (Figs 2 and 6) are identical within experimental error (pK $9{\cdot}0 \pm 0{\cdot}1$). As has been shown by Tanford (1961), identical variations of a structural parameter and of the binding of one proton requires that there is a complete shift of one conformation to the other. Therefore, when they have bound neither inhibitor, nor proton, essentially all the enzyme molecules have the same conformation, referred to here as E'. On binding indole the structure of the protein becomes that of the E species, as indicated by Figure 4.

Consequently Ac-δ-CT seems to assume two discrete conformational states, one in which either a proton is bound to the N-terminal amino group of isoleucine 16 or an

indole molecule is bound to the active site, (or both), and one in which neither proton nor inhibitor are bound, and we are led to the conclusion that the equilibrium between the two structures is completely shifted by one or the other ligand.

(b) *Binding interactions between protons and indole*

The scheme summarizing the experimental observations (scheme IV) involves multiple equilibria, and is a simplification of the minimum scheme in which single equilibria are considered. This more comprehensive scheme is:



similar to that proposed by Himoe *et al.* (1967). It implies that the E and E′ species are in equilibrium, whether or not they have bound a ligand.

The isomerization equilibrium constants are related to the "true" dissociation constants and to the experimental apparent dissociation constant, K_a, by the relationships

$$L = \frac{E'}{E} = \frac{K_a}{K'_a} \quad \text{and} \quad L' = \frac{E'H}{EH} = \frac{K_a}{K''_a}$$

where K'_a and K''_a are respectively the ionization constants of the isoleucine 16 amino group in the E and in the E′ state. If one admits the independence of the indole and of the proton binding sites in the E state, a reasonable assumption since this conformation is assumed by the enzyme on binding either a proton or indole, one has:

$$K'_a = \frac{(EI)\,(H)}{(EIH)} = \frac{(E)\,(H)}{(EH)}.$$

This is equivalent to saying that the ionization constant of the amino group is the same in the free enzyme and in the enzyme–indole complex when the protein is in the E conformation. Then $L = K_a/K'_a$. The first equilibrium constant, L, can be deduced from the values obtained for K_a and K'_a: taking $K_a = 10^{-9}$ M and $K'_a = 3{\cdot}5 \times 10^{-11}$ M, the average value of the polarimetric and of the potentiometric measurements, one finds $L \simeq 10^{-9}/3{\cdot}5 \times 10^{-11} \simeq 30$. In the non-protonated state and in the absence of inhibitor, the enzyme molecules are therefore mostly in the E′ conformation (97% of E′ *versus* 3% of E).

The second equilibrium constant, L', can be obtained approximately if one considers that in the E′ conformation the electrostatic interaction between the aspartic

acid 194 and the isoleucine 16 residues no longer exists, as suggested by Sigler *et al.* (1968); if there is no more interaction the amino group can be expected to have a close to normal ionization, i.e. a pK around 7·5 at 25°C or 7·8 at 15°C ($K''_a \simeq 1{\cdot}6 \times 10^{-8}$ M). The isomerization constant L' would then be $L' \simeq 10^{-9}/1{\cdot}6 \times 10^{-8} \simeq 1/16$. This value corresponds to an equilibrium in which the protonated enzyme molecules would be distributed between the E and E′ conformation in the 94%–6% ratio. This is still in a range compatible with the finding of superimposable potentiometric and polarimetric titration curves, but a somewhat higher pK for the amino group in the E′ state would hardly allow this agreement; binding of indole to E′H species would be expected to shift it to the EH form, as observed for the E′ conformation, and change the 94%–6% ratio to close to the 100%–0%. The replacement of six per cent of an E′ form by an E form corresponds to an experimentally measured optical rotation change of about two millidegrees, which is the upper limit of the experimental variations of the measurements. Therefore if the amino group had an abnormally high ionization constant in the E′ conformation of the enzyme, the present data would most likely show it and we believe that these data confirm the suggestions made by Sigler *et al.* (1968) that the electrostatic interaction between isoleucine 16 and aspartic acid 194 does not exist in the "high pH conformation" of chymotrypsin. When Ac-δ-CT goes from the E′ to the E state, on binding indole, the ionization constant of the amino group of its isoleucine 16 residue is shifted several hundred-fold, its pK passing from 10·5 to below 8. This seems a good example of binding interaction between a non-polar molecule and a charged group through a conformational change of the protein.

As has been seen in Figure 3 the fixation of indole is enhanced by the fixation of the proton, both effects being mutual. However, in the state of knowledge of the system we do not know the binding constant of indole to the E′ conformation of the enzyme and its change between E′ and E. The apparent K_1 at high pH is the over-all dissociation constant corresponding to an isomerization step and a binding step since indole converts the structure of the enzyme from E′ to E. A lower limit of the dissociation constant of indole to E′, $K_1'' = (E')\,(I)/(E'I)$, can be set from the present data. As seen in Figure 4, from pH 9 to 11 the specific optical rotation of the enzyme at indole saturation is the same, and is identical to that at pH 7 with or without indole (Figs 2 and 3). Binding of a proton to the enzyme–indole complex does not change the structure of the protein beyond an experimentally observable shift in optical rotation. As discussed above this requires that much less than ten per cent of the enzyme molecules could be converted from the E′ to the E form, when the enzyme–indole complex binds a proton, and therefore that at indole saturation less than ten per cent of the molecules are E′I and more than 90% are EI. This means that K''_1 is more than ten times larger than $K'_1 : K''_1 > 0{\cdot}15$ M. This is also in agreement with the ideas put forward by Sigler *et al.* (1968) who propose that the substrate is totally unable to bind at the active site of the zymogen, the zymogen and the E′ structure being close to one another. This inability to bind the substrate or a specific inhibitor would also be an example of the "exclusive binding" concept of the allosteric model, the E′ structure being equivalent to the T state of this model.

(c) *The acidic counterpart of the alkaline transition*

The structural change that is described here has been hypothetically related to the internal salt bridge evidenced by the crystallographic data of Matthews *et al.* (1967). If this is a correct explanation the deionization of a carboxylate residue should have a

similar structural consequence to that of the N-terminal isoleucine amino group, and should be prevented by binding an inhibitor or a substrate to the active site.

These two consequences are shown to be in agreement with the studies of Parker & Lumry (1963) on chymotrypsin and of Béchet & d'Albis (1969) on the homologous enzyme trypsin. Parker & Lumry (1963) have shown that a structural transition is absent when a specific acyl group, acetyl tyrosine, is bound to the active site of the enzyme. Therefore one can conclude that a carboxyl group has the same role as the isoleucine 16 amino group and it is most reasonable to consider this group to be the aspartic acid residue 194 adjacent to the active serine (Matthews *et al.*, 1967).

Recently d'Albis & Béchet (1967), and Béchet & d'Albis (1969) have carried out an extensive study on the enzyme–inhibitor system trypsin–benzamidine, both by polarimetry and potentiometry. They arrive at the conclusions that two ionizable groups (pK values around 4 and 10) are involved in the locking of the enzyme active structure and that these pK values are both shifted to more extreme values when the specific inhibitor benzamidine binds to the active site. These data strongly support the hypothesis of the two-state equilibrium and the double dependency of this equilibrium on the ion pair aspartic acid–isoleucine and on the presence of a specific ligand on the active site.

(d) *Influence of the E $\rightarrow$ E′ transition on the enzyme activity*

Himoe, Parks & Hess (1967) showed that the catalytic activity of δ-chymotrypsin is not changed towards a specific substrate such as N-acetyl tryptophan amide in the pH range where the enzyme goes from the E to the E′ structure. These authors observed only a decrease of the affinity constant of δ-chymotrypsin for its substrate and they proposed that this fact was linked to the pK shift of the conformation controlling ionizing group. However the activity of the enzyme at those pH values where the isoleucine does not protonate (pH $\gg$ pK_a) is not known. The present results suggest that a specific substrate should behave like indole, i.e. change the structure of the enzyme from the E′ to the E state, and bring the enzyme to its normally active conformation. This is seen with the ester substrates (Bender *et al.*, 1964 and references therein) for which there is no pH dependency, and also with amide substrates such as N-acetyl tryptophan amide, towards which Ac-δ-CT is active up to pH 12 (B. Labouesse, unpublished experiments). Whether the enzyme binds a specific inhibitor or a specific substrate it is able to assume the proper active conformation without help of the aspartic acid–isoleucine interaction; however the absence of this interaction is reflected in a greatly increased K_m or K_i; if, as proposed by Sigler *et al.* (1968) and McConn *et al.* (1969), the aspartic acid side chain sits inside the active site in the E′ state, this side chain is able to switch back towards the non-protonated isoleucine in the enzyme. This would imply that the E′ structure differs somewhat from that of the zymogen, since the zymogen cannot accept the substrate in a proper way, and the activation mechanism as proposed by Sigler *et al.* (1968) must be refined, and include at least one state between the zymogen and the E structure.

If the substrate possesses the specific stereo-conformation fitting with the active conformation of the enzyme, one can ask the question whether or not the E′ state will be operating catalytically, since it is essentially converted to E. Now if the substrate does not have this proper geometry one can imagine that it cannot push back the aspartic 194 side chain and cannot help the active histidine–serine pair to resume its best position. The E′ state would then be potentially less active than the E state. If

the enzyme, whose isoleucine 16 amino group has been blocked by reacetylation, is compared to both the E′ state and the zymogen, (since these three proteins have the same polarimetric properties (McConn *et al.*, 1969; C. Ghélis, unpublished experiments)) it most likely resembles E′ more than the zymogen; this reacetylated enzyme does indeed hydrolyse *p*-nitrophenyl acetate (Ghélis *et al.*, 1967) whereas the zymogen does not, but its rate of hydrolysis is much slower than that of the active enzyme E (Ghélis, to be published). Therefore we suggest that E′ is not inactive but is potentially less active than E on non-specific substrates and that it has to be ignored as far as specific substrates are concerned since it is isomerized into E by these substrates.

(e) *Consequence of the E′ → E transition*

At least two consequences can be inferred from the E′ → E transition.

(1) As we have seen, the conversion of the molecules E′ into the E state can be brought about by binding of a specific inhibitor such as indole. If the isomerization process is slow enough to allow the inhibitor to leave the active site without reversion of the E structure to E′, the net result of inhibitor binding would be an increase of the actual concentration of active enzyme molecules and not a decrease; therefore the apparent activity of the enzyme on poorly specific substrates should increase at high pH in the presence of non-saturating concentrations of indole, instead of decrease, and this specific inhibitor should then act as an activator.

(2) The E′ → E conversion can equally well be forced by isoleucine 16 protonation. If this process is slow, as it seems to be (Garel, unpublished data), the catalytic activity should show a lag phase when the enzyme is brought from high pH to neutral pH before reaching its normal steady state. This lag phase is directly related to the relaxation time of the transition, and corresponds to the time necessary for converting the E′ form into the E form by protons and by substrate.

Obviously much work is needed to solve the linked problems of conformational change, ligand affinity changes and active site integrity that have been raised in these different studies and a partial answer to these questions will be given by the kinetic investigations of individual isomerization steps under study. However, data such as those presented here, as well as those of Sigler *et al.* (1968), show that a simple protein like chymotrypsin, whose three-dimensional structure and many properties are known, may eventually lead to a good model of the binding interactions that are fundamental to all regulated enzymes.

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